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Pax7 Is Required for the Specification of Myogenic Satellite Cells

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Summary

The paired box transcription factor *Pax7* was isolated by representational difference analysis as a gene specifically expressed in cultured satellite cell-derived myoblasts. In situ hybridization revealed that *Pax7* was also expressed in satellite cells residing in adult muscle. Cell culture and electron microscopic analysis revealed a complete absence of satellite cells in *Pax7*$^{-/-}$ skeletal muscle. Surprisingly, fluorescence-activated cell sorting analysis indicated that the proportion of muscle-derived stem cells was unaffected. Importantly, stem cells from *Pax7*$^{-/-}$ muscle displayed almost a 10-fold increase in their ability to form hematopoietic colonies. These results demonstrate that satellite cells and muscle-derived stem cells represent distinct cell populations. Together these studies suggest that induction of Pax7 in muscle-derived stem cells induces satellite cell specification by restricting alternate developmental programs.

Introduction

Muscle satellite cells represent a distinct lineage of myogenic progenitors responsible for the postnatal growth, repair, and maintenance of skeletal muscle (reviewed in Seale and Rudnicki, 2000). At birth, satellite cells account for ~30% of sublaminar muscle nuclei in mice followed by a decrease to <5% in a 2-month-old adult (Bischoff, 1994). This decline in satellite cell nuclei reflects the fusion of satellite cells during the postnatal growth of skeletal muscle (Gibson and Schultz, 1983). Satellite cells were originally defined on the basis of their unique position in mature skeletal muscle and are closely juxtaposed to the surface of myofibers such that the basal lamina surrounding the satellite cell and its associated myofiber is continuous (Bischoff, 1994).

In mice >2 months of age, satellite cells in resting skeletal muscle are mitotically quiescent and are activated in response to diverse stimuli, including stretching, exercise, injury, and electrical stimulation (Schultz et al., 1985; Appell et al., 1988; Rosenblatt et al., 1994; reviewed in Bischoff, 1994). The descendents of activated satellite cells, called myogenic precursor cells, undergo multiple rounds of cell division before fusion with new or existing myofibers. The total number of quiescent satellite cells in adult muscle remains constant over repeated cycles of degeneration and regeneration, suggesting that the steady-state satellite cell population is maintained by self-renewal (Gibson and Schultz, 1983; Schultz and Jaryszak, 1985; Morlet et al., 1989). Therefore, satellite cells have been suggested to form a population of monopotential stem cells that are distinct from their daughter myogenic precursor cells as defined by biological and biochemical criteria (Grounds and Yablonka-Reuveni, 1993; Bischoff, 1994).

Satellite cells clearly represent the progenitors of the myogenic cells that give rise to the majority of the nuclei within adult skeletal muscle. However, recent studies have identified a population of pluripotential stem cells, also called side-population (SP) cells, in adult skeletal muscle. Muscle-derived SP cells are readily isolated by fluorescence-activated cell sorting (FACS) on the basis of Hoechst dye exclusion (Gussoni et al., 1999; Jackson et al., 1999). Purified SP cells derived from muscle exhibit the capacity to differentiate into all major blood lineages after tail vein injection into lethally irradiated mice (Jackson et al., 1999). Of particular significance is the observation that transplanted SP cells isolated from bone marrow or muscle actively participate in myogenic regeneration. However, only muscle-derived SP cells appear to give rise to myogenic satellite cells (Gussoni et al., 1999). In addition, SP cells convert to desmin-expressing myoblasts after exposure to appropriate cell culture conditions (Gussoni et al., 1999). However, whether SP cells are equivalent to satellite cells, are progenitors for satellite cells, or represent an entirely independent cell population has remained unclear.

The gene expression profile of quiescent satellite cells and their activated progeny is largely unknown. Quiescent satellite cells express the *c-Met* receptor (receptor for hepatocyte growth factor) and M-cadherin protein (Irintchev et al., 1994; Cornelison and Wold, 1997). Activated satellite cells upregulate *MyoD* or *Myf5* before entering S-phase (Cornelison and Wold, 1997). Proliferating myogenic precursor cells, the daughter cells of satellite cells, express desmin, Myf5, MyoD, and other myoblast specific markers (George-Weinstein et al., 1993; Cornelison and Wold, 1997). Nevertheless, the paucity of cell-lineage specific markers has been a significant impediment to understanding the relationship between satellite cells and their progeny.

Our poor understanding of molecular events responsible for satellite cell development and activation indicated the use of a PCR-based subtractive hybridization approach (Hubank and Schatz, 1994) to identify tissue-specific genes expressed in the satellite cell myogenic lineage. Results from this analysis identified several myoblast-specific genes potentially involved in satellite cell function. *Pax7* was selected for further analysis based

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on the established role of the closely related Pax3 protein in regulating the developmental program of embryonic myoblasts (Maroto et al., 1997; Tajbakhsh et al., 1997). In this article, we demonstrate a unique requirement for Pax7 in the specification of myogenic satellite cells.

Results

Identification of Genes Expressed in Satellite Cell-Derived Myoblasts

To gain insight into the developmental program responsible for the differentiation and activation of skeletal muscle satellite cells, representational difference analysis (RDA) of cDNAs was employed to identify genes expressed specifically in satellite cell-derived myoblasts (Hubank and Schatz, 1994). This analysis resulted in the identification of 17 distinct products corresponding to 12 known and 5 potentially novel genes by searching GenBank (NCBI) using the FASTA program (unpublished data). RDA clone *dp3–7* encoded a fragment from within the *Pax7* mRNA. Pax7 is a member of the paired-box family of transcription factors that play important regulatory roles in the development of diverse cell lineages (Mansouri, 1999). Therefore, a full-length 4.3-kb *Pax7* cDNA was isolated from an adult mouse skeletal muscle cDNA library (Clontech) to facilitate further analyses (NCBI accession number AF254422).

Pax7 is Specifically Expressed in Proliferating Myoblasts

Detailed expression analysis of the distribution of *Pax7* mRNA was conducted using Northern blot analysis (Figure 1). These analyses demonstrated that *Pax7* was expressed exclusively in proliferating primary myoblasts, with comparable levels of expression in both wild-type and *Myo*$^{-/-}$ cultures (Figure 1A). However, *Pax7* mRNA was downregulated after myogenic differentiation (Figure 1A). Furthermore, *Pax7* was not expressed at detectable levels in a variety of nonmuscle cell lines (Figure 1B). Rather, *Pax7* was strictly expressed in myogenic cells, including low levels in proliferating C2C12 mouse myoblasts, which is a continuous cell line originally derived from satellite cells (Figure 1B). In addition, *Pax7* mRNA was not detectable in 20 μg of total RNA from several adult mouse tissue samples (Figure 1C). Analysis of poly(A)$^+$ RNA from select mouse tissues revealed expression of *Pax7* at low levels only in adult skeletal muscle (not shown). Therefore, in adult mice, *Pax7* expression appears specific to the satellite cell myogenic lineage.

Pax7 Is Expressed in Satellite Cells

To localize *Pax7* mRNA in skeletal muscle, we performed in situ hybridization on fresh frozen sections of tibialis anterior and gastrocnemius muscles from wild-type (Balb/c), *MyoD*$^{-/-}$, *mdx*, and compound mutant *mdxMyoD*$^{-/-}$ animals. Interestingly, *Pax7* mRNA was associated with a subset of nuclei in discrete peripheral locations within undamaged wild-type (Figures 2A and 2C) and *MyoD*$^{-/-}$ (not shown) skeletal muscle. Propidium iodide (PI) staining was used to identify all nuclei within skeletal muscle, thereby allowing for the enumeration of *Pax7*-positive cells (Figures 2B, 2D, and 2F). The in situ hybridization was repeated on muscle sections from

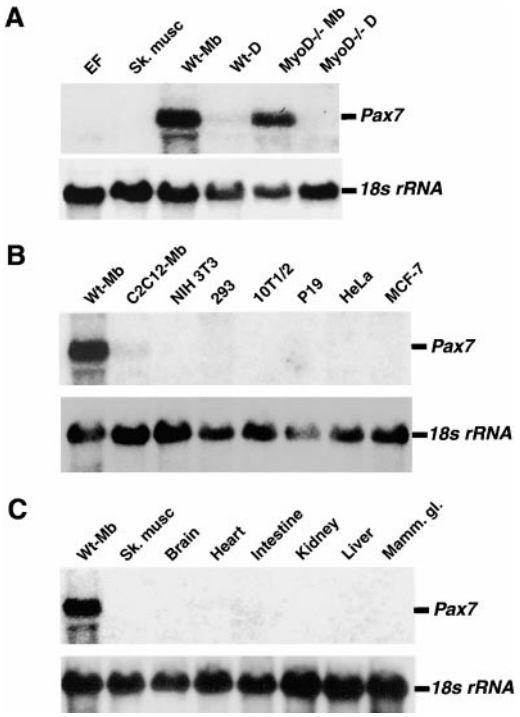

Figure 1. *Pax7* Is Expressed Specifically in Proliferating Myoblasts

(A) *Pax7* was expressed at high levels in proliferating wild-type myoblasts (Wt-Mb) and *MyoD*-deficient cells (MyoD$^{-/-}$ Mb) cells and downregulated in response to differentiation conditions (Wt-D and MyoD$^{-/-}$ D).

(B) Expression of *Pax7* was specific to myogenic cells, with low levels detected in C2C12 myoblasts.

(C) *Pax7* was not detected in RNA from a panel of tissues.

three independent mice by using three separate sequences as anti-sense cRNA probes to verify the expression patterns described. Approximately 5% of muscle nuclei (including satellite cell nuclei and myonuclei) were associated with *Pax7* expression in adult wild-type muscle. By contrast, the number of *Pax7*-positive cells increased to 22% in *MyoD*$^{-/-}$ muscle. The increased expression of *Pax7* in *MyoD*$^{-/-}$ muscle strongly supports the notion that *Pax7* is expressed in satellite cells, because previous work has revealed that *MyoD*-deficient muscle contains increased numbers of satellite cells (Megeney et al., 1996). At high magnification (200×), *Pax7* appeared to be expressed in cells residing beneath the basal lamina of wild-type muscle fibers in positions characteristic for quiescent satellite cells (Figure 2C).

To determine whether *Pax7* was upregulated in regenerating skeletal muscle, we analyzed 3-week-old *mdx* and compound mutant *mdxMyoD*$^{-/-}$ skeletal muscle by in situ hybridization. Lack of dystrophin protein causes *mdx* muscle to undergo repeated cycles of muscle degeneration and regeneration (Sicinski et al., 1989). As predicted, given the high levels of expression in cultured satellite cell-derived myoblasts, *Pax7* was widely expressed in regenerating areas of *mdx* and *mdxMyoD*$^{-/-}$ skeletal muscle (Figure 2E). Centrally located nuclei within muscle fibers of *mdx* (Figure 2E), *MyoD*$^{-/-}$ (not shown), and *mdxMyoD*$^{-/-}$ (not shown) muscle were also associated with *Pax7* expression, suggesting that recently activated and fusing myogenic precursors express *Pax7*. Lastly, a similar distribution of immunoreactive nuclei was observed in muscle sections stained with

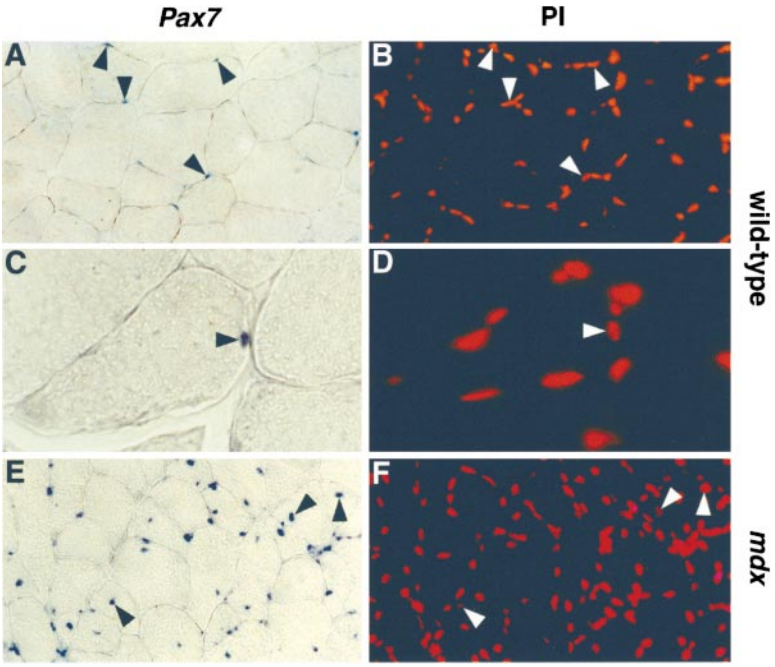

Figure 2. Expression of *Pax7* in Muscle Satellite Cells

(A) In situ hybridization revealed that *Pax7* mRNA was expressed at a frequency and location consistent with specific expression in satellite cells and myogenic precursor cells.

(B) *Pax7* expression was associated with PI-positive nuclei (40× magnification).

(C and D) High magnification (200×) of *Pax7*-expressing cell in wild-type muscle was characteristic of a satellite cell residing beneath the basal lamina.

(E and F) Increased numbers of cells expressed *Pax7* in regenerating *mdx* muscle (40×). Black and white arrowheads indicate cells stained positive for *Pax7* mRNA and PI-positive nuclei, respectively.

anti-Pax7 antibody (Developmental Studies Hybridoma Bank). Taken together, these results support the notion that *Pax7* is expressed within the satellite cell lineage. Therefore, these results raise the hypothesis that Pax7 is required for the ontogeny or function of muscle satellite cells.

Skeletal Muscle Deficiency in Pax7 Mutant Animals

To evaluate possible roles for Pax7 in the formation or function of satellite cells, we examined skeletal muscle from mice carrying a targeted null mutation in *Pax7* (Mansouri et al., 1996b). Mice deficient for *Pax7* express muscle-specific markers, including *MyoD* and *Myf5*, in a normal spatial and temporal pattern within the developing myotome (Mansouri et al., 1996b). However, *Pax7*$^{-/-}$ mice were significantly smaller than their wild-type and heterozygous counterparts (Figure 3A). The body weight of *Pax7*$^{-/-}$ mice at 7 days of age was 50% reduced in comparison with wild-type littermates ($n = 20$). This weight differential increased with age such that at 2 weeks of age, mutant animals were ~33% the weight of wild-type littermates. As previously reported, *Pax7* mutant animals failed to thrive and usually died within 2 weeks after birth (Mansouri et al., 1996b). In addition, we observed that mutant mice exhibited muscle weakness characterized by an abnormal gait and splayed hind limbs (not shown). Light microscopic analysis of hematoxylin-eosin (HE)-stained lower hind limb skeletal muscle (below the knee) of 1-week-old wild-type (Figure 3B) and *Pax7*$^{-/-}$ (Figure 3C) animals revealed a 1.5-fold reduced diameter of *Pax7* mutant fibers ($n = 100$ fibers). However, the overall organization of muscle fibers was not affected. Moreover, the diaphragms of 7-day-old *Pax7*$^{-/-}$ mice (Figure 3E) were notably thinner than those of their wild-type littermates (Figure 3D). Therefore, the markedly decreased muscle mass and reduced fiber caliber of *Pax7* mutant muscle suggested that the postnatal growth phase of skeletal muscle normally mediated by satellite cells was deficient in the absence of Pax7.

Absence of Satellite Cell-Derived Myoblasts from *Pax7*$^{-/-}$ Muscle

To gain insight into satellite cell function in *Pax7* mutant mice, we cultured primary cells directly from the muscle of 7- to 10-day-old wild-type mice and *Pax7*$^{-/-}$ littermates in five independent experiments. After 2 days in culture, many bursts of satellite cell-derived myoblasts were readily identified in wild-type primary cultures on the basis of morphological criteria (Figure 4A) and immunocytochemistry by using both anti-desmin and anti-c-Met antibodies that mark satellite cell-derived myoblasts (Figures 4B–4E). Strikingly, no myoblasts were identified in mutant cultures, which instead were uniformly composed of fibroblasts and adipocytes, as identified by morphological and immunochemical criteria (Figures 4F–4J).

To further investigate whether myogenic cells were present in postnatal *Pax7* mutant muscle, individual muscle fibers from 7- to 10-day-old wild-type mice and *Pax7*$^{-/-}$ littermates were isolated in five independent experiments and cultured in methylcellulose stem-cell medium. Methylcellulose stem-cell medium readily promotes the activation, migration, and proliferation of satellite cells associated with muscle fibers (A. Asakura and M.A.R., unpublished observation). After 48 and 72 hr in culture, satellite cells associated with wild-type fibers generated distinct bursts of desmin-expressing myogenic cells. By contrast, *Pax7* mutant muscle fibers did not give rise to any mononuclear cells. After 2 weeks in culture, large colonies of fully contractile myosin heavy chain (MHC)-expressing myotubes were present in cultures of wild-type but not *Pax7*$^{-/-}$ fibers (not shown). Therefore, these results suggest that satellite cells do not exist or, alternately, that they fail to proliferate in the absence of Pax7.

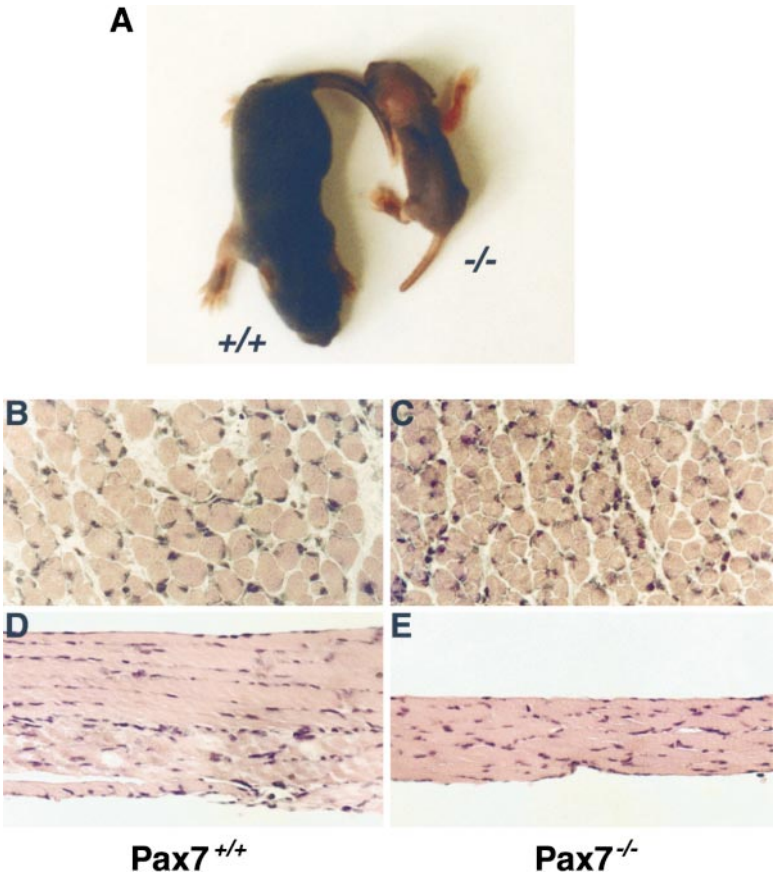

Figure 3. Skeletal Muscle Deficiency in *Pax7*$^{-/-}$ Mice

(A) Seven-day-old *Pax7* mutant animals were approximately one-half the weight of wild-type animals and had splayed hind limbs and an abnormal gait.

(B and C) HE-stained tibialis anterior muscle sections (40×) revealed a normal histological appearance of *Pax7* mutant muscle (C), but fiber diameter was reduced 1.5-fold compared with that of wild-type muscle (B).

(D and E) The diaphragm of a mutant animal (E) shown here in cross-section was significantly thinner than that in wild-type animals (D) (40×).

Complete Ablation of Satellite Cells in *Pax7*$^{-/-}$ Muscle

To determine whether or not satellite cells were present in mutant animals, we used transmission electron microscopy (TEM) to analyze skeletal muscle from wild-type and *Pax7*$^{-/-}$ mice. Biopsies from gastrocnemius muscle of three 7- to 10-day-old wild-type mice and mutant littermates were analyzed by TEM. For each sample, 100 peripheral sublaminar nuclei were analyzed and identified as either satellite cell or myofiber nuclei. Criteria for the identification of satellite cells consisted of the following: a plasma membrane separating the satellite cell from its adjacent muscle fiber, an overlying basal lamina continuous with the satellite cell and associated fiber, and the characteristic heterochromatic appearance of the nucleus (reviewed in Bischoff, 1994).

Satellite cells were readily identified in wild-type muscle and comprised 25% of peripheral sublaminar nuclei (n = 300) (Figures 5A–5D). By contrast, satellite cells could not be identified in >300 sublaminar nuclei examined from mutant muscles (Figures 5E and 5F).

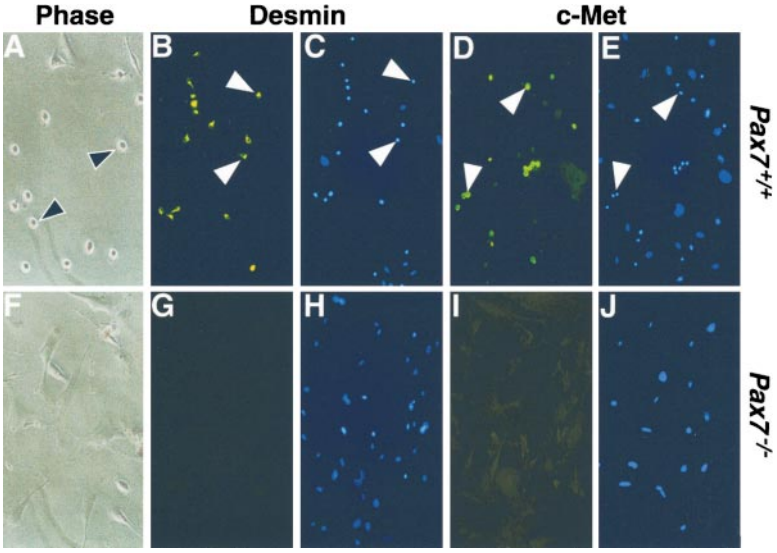

Figure 4. Absence of Myoblasts in Cultures Derived from *Pax7*$^{-/-}$ Muscle

Primary cell cultures were analyzed by phase microscopy (A and F) and by immunocytochemistry with anti-desmin (B and G) and anti-c-Met (D and I) antibodies. Cells stained with antibodies were counterstained with Hoechst 33342 (C, E, H, and J) to show all nuclei. Black arrowheads indicate satellite cell-derived myoblasts; white arrowheads indicate immunoreactive cells and corresponding nuclei.

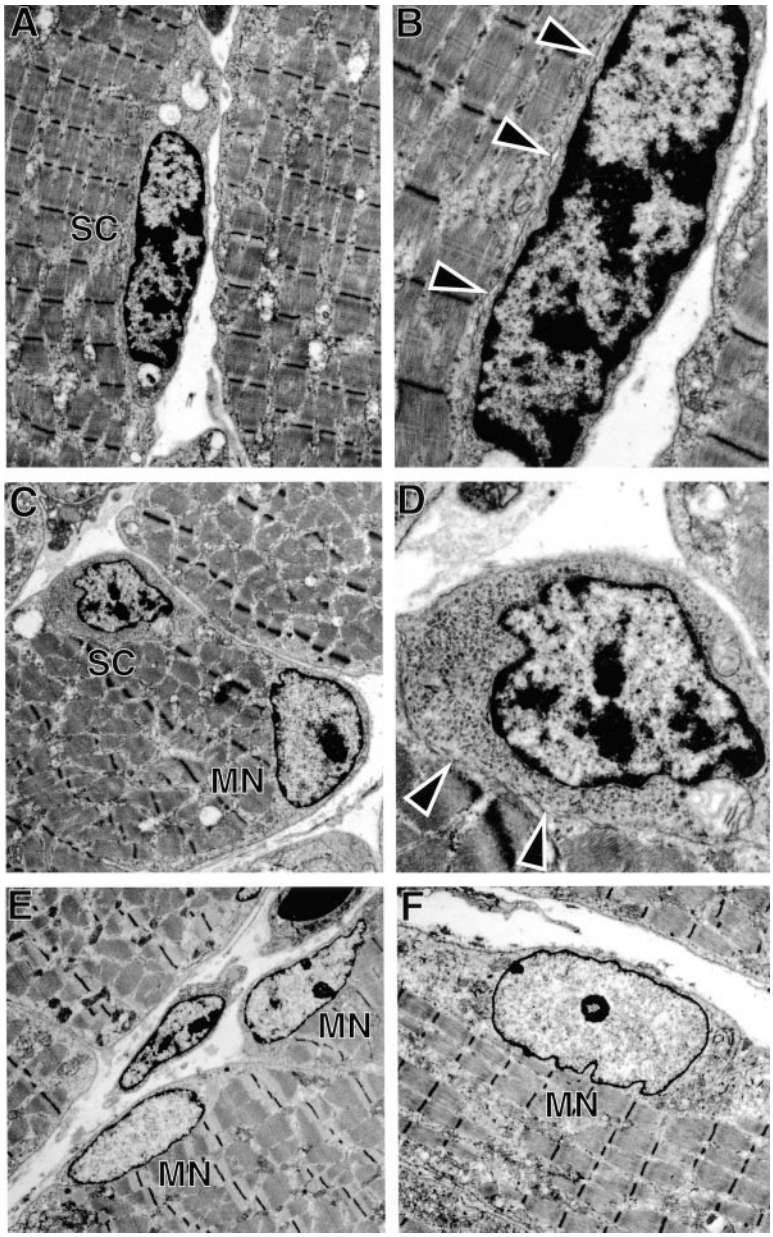

Figure 5. Complete Ablation of Satellite Cells in *Pax7*$^{-/-}$ Muscle

Transmission electron micrographs of 7- to 10-day-old *Pax7*$^{+/+}$ (A through D) and *Pax7*$^{-/-}$ (E and F) muscle.

(A and C) Satellite cells (SC) are readily identified in *Pax7*$^{+/+}$ muscle (7500×).

(B and D) High magnification of satellite cells clearly revealed the plasma membrane (black arrowheads) separating the satellite cell from its adjacent myofiber, the continuous basal lamina surrounding the satellite cell and myofiber, and the heterochromatic appearance of the nucleus (20,000×).

(E and F) Myonuclei (fiber nuclei) (MN) but not satellite cells were present in *Pax7* mutant muscles. Other ultrastructural differences were not detected.

Furthermore, satellite cells were not found in mutant muscle from E18 embryos (18 days post-coitum; not shown). Therefore, in the absence of Pax7, complete ablation of muscle satellite cells was observed. The failure of muscle satellite cells to form in *Pax7*$^{-/-}$ muscle thus unequivocally establishes an essential role for Pax7 in the ontogeny of the satellite cell lineage.

Muscle-Derived SP Cells are Present in *Pax7* Mutant Muscle

To investigate the relationship between satellite cells and muscle-derived pluripotent stem cells, we performed FACS analysis of cells isolated from wild-type and *Pax7*$^{-/-}$ muscle. Recent work has identified a population of pluripotent stem cells (also called side-population [SP] cells) in skeletal muscle as defined by Hoechst 33342 dye exclusion (Gussoni et al., 1999; Jackson et al., 1999). Cell suspensions isolated directly from 1-week-old skeletal muscle were stained with Hoechst dye in the presence or absence of verapamil. The SP cell population is sensitive to verapamil, which is thought to prevent dye efflux through the inhibition of mdr (multidrug resistant)-like proteins (Goodell et al., 1996, 1997). On the basis of results from three independent trials with six 7- to 10-day-old *Pax7*$^{-/-}$ and wild-type animals, the proportion of muscle SP cells was unaffected by the absence of Pax7 (Figures 6A–6D). The relative proportion of SP cells in wild-type (1.8%) (Figure 6A) versus Pax7 mutant (1.5%) (Figure 6C) muscle did not differ significantly. Taken together, these data indicate that muscle satellite cells are either a population distinct from muscle SP cells or, alternately, represent only a small subpopulation of muscle SP cells.

Stem Cells Derived From *Pax7*$^{-/-}$ Exhibit Markedly Increased Hematopoietic Potential

To characterize the differentiation potential of *Pax7*-deficient stem cells, we assayed dissociated muscle

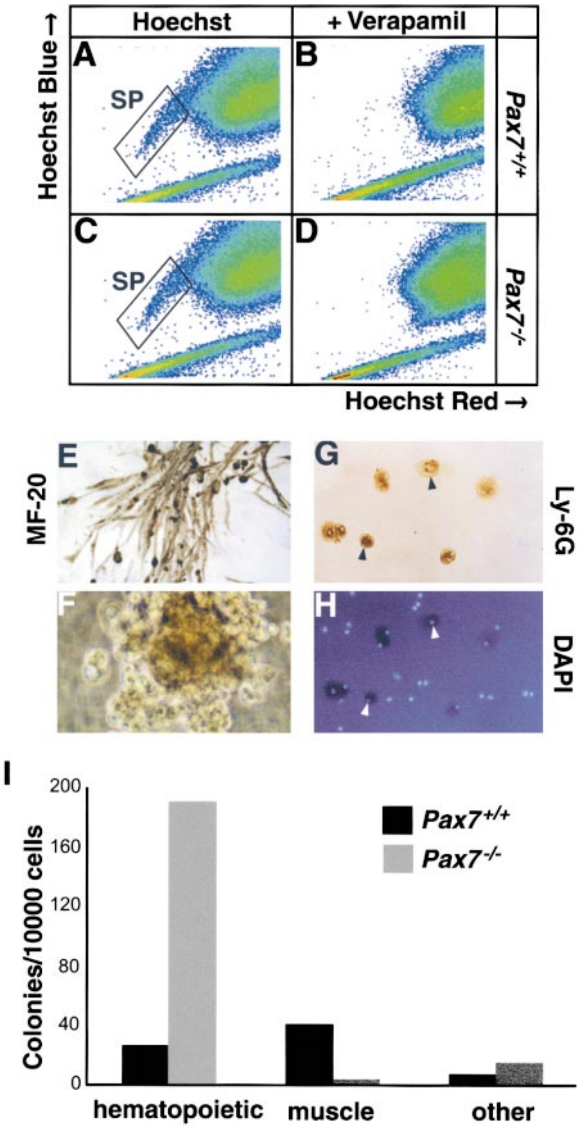

Figure 6. Enhanced Hematopoietic Potential of *Pax7*$^{-/-}$ Muscle–Derived Pluripotent Stem Cells

(A through D) FACS analysis of Hoechst-stained muscle-derived cells demonstrated approximately equal numbers of verapamil-sensitive SP cells in both *Pax7*$^{+/+}$ (A and B) and *Pax7*$^{-/-}$ (C and D) muscles.

(E) MHC-positive muscle colonies predominate in stem-cell medium/methylcellulose cultures of *Pax7*$^{+/+}$ muscle cells.

(F through H) *Pax7*$^{-/-}$ muscle cells have increased hematopoietic potential (F) and generate granulocyte and monocyte colonies verified by Ly-6G immunoreactivity (G and H).

(I) Colony-forming assay of muscle cells cultured in stem-cell medium/methylcellulose over a period of 2 weeks demonstrated almost a 10-fold increase in hematopoietic potential of *Pax7* mutant stem cells. Other cells represent both fibroblasts and adipocytes.

cells from 7- to 10-day-old *Pax7*$^{-/-}$ and wild-type animals for colony formation in methylcellulose stem-cell medium, which allows the growth of muscle as well as hematopoietic colonies (A. Asakura and M. A. R., unpublished data). Seven independent experiments were analyzed in which 10,000 cells from both wild-type and *Pax7*$^{-/-}$ muscle were cultured. Hematopoietic colonies included granulocytic and monocytic cells and were present in both wild-type and mutant cultures on the basis of immunoreactivity with Ly-6G (Figures 6G and 6H) and Integrin α_M chain (not shown). Ly-6G is a cell surface antigen, which is expressed exclusively in granulocyte and monocyte lineages (Fleming et al., 1993). Integrin α_M chain, also known as MAC-1, is expressed on granulocytes, macrophages, and natural killer cells (Leenen et al., 1994). Wild-type cultures were predominantly composed of contractile muscle colonies reactive with antibody to MHC (Figure 6E). By contrast, *Pax7*$^{-/-}$ cultures exhibited a markedly increased potential for hematopoietic differentiation (Figure 6F) and generated ~10-fold the number of hematopoietic colonies compared with wild-type cultures (Figure 6I). To rule out the possibility that the presence of differentiating muscle cells was inhibiting hematopoietic differentiation in wild-type cultures, we analyzed mixed cultures of *Pax7*$^{-/-}$ and wild-type cells. Results from these experiments showed that hematopoietic colony formation was not adversely affected by differentiating myocytes (not shown).

The colony-forming assays summarized in Figure 6I depict the average number of hematopoietic, skeletal myocyte, and other (e.g., fibroblast and adipocyte) colonies from seven independent isolations performed in triplicate. Therefore, stem cells isolated from muscle lacking *Pax7* exhibited a strongly increased propensity toward hematopoietic differentiation and were incapable of forming adult myoblasts. Importantly, highly purified SP cells from wild-type muscle convert to myoblasts under the appropriate culture conditions (Gussoni et al., 1999). Taken together, these results suggest the hypothesis that induction of *Pax7* in pluripotent muscle-derived stem cells directs the specification of satellite cells through restriction of developmental potential (Figure 7).

Discussion

Pax7 was molecularly cloned by RDA in a screen designed to identify genes specifically expressed in the muscle satellite cell lineage. On the basis of Northern blot analysis, expression of *Pax7* was confined to proliferating myoblasts and was strongly downregulated during terminal differentiation (Figure 1). In situ hybridization studies revealed the apparent localization of *Pax7* mRNA to satellite cells and their daughter myogenic precursor cells (Figure 2). Analysis of postnatal skeletal muscle from *Pax7*$^{-/-}$ animals revealed a complete absence of myogenic satellite cells as determined by primary cell culture and TEM (Figures 4 and 5). However, FACS analysis indicated that the proportion of SP cells was unaffected by the absence of Pax7 (Figure 6). Importantly, cells cultured from wild-type muscle efficiently gave rise to MHC-expressing myocyte colonies, whereas *Pax7*$^{-/-}$ muscle cells were unable to form myoblasts but exhibited a 10-fold increase in hematopoietic potential (Figure 6). Taken together, these data implicate Pax7 in the specification of myogenic satellite cells from uncommitted progenitors in skeletal muscle (Figure 7).

Pluripotential muscle-derived stem cells purified by FACS exhibit the capacity to efficiently reconstitute the marrow compartment and appear to give rise to muscle satellite cells after intravenous injections in mice (Gussoni et al., 1999; Jackson et al., 1999). Moreover, purified

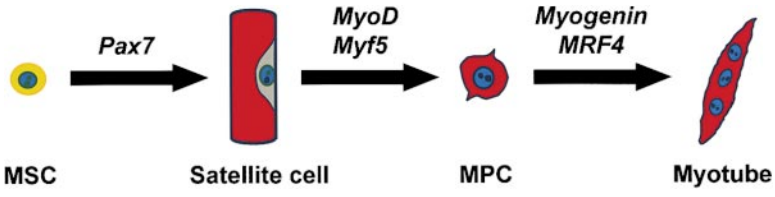

Figure 7. Role for Pax7 in the Specification of Satellite Cells

Muscle-derived pluripotent stem cells primarily give rise to myoblasts when cultured in stem-cell medium. By contrast, *Pax7*$^{-/-}$ muscle stem cells exhibit almost a 10-fold increase in propensity toward hematopoietic differentiation and are incapable of forming adult myoblasts. These data therefore implicate Pax7 in regulating the specification of adult muscle satellite cells by restricting the fate of pluripotent stem cells. Taken together, these experiments suggest the following hypothesis. Pluripotent stem cells (MSC) within muscle represent the progenitors of sublaminar satellite cells that are specified following induction of *Pax7*. Satellite cells are subsequently activated in response to physiological stimuli to generate daughter myogenic precursor cells (MPC) before terminal differentiation into new or previously existing fibers.

muscle-derived SP cells convert to desmin-expressing myoblasts in response to appropriate cell culture conditions (Gussoni et al., 1999). On the basis of our results demonstrating normal numbers of SP cells and the complete absence of satellite cells in *Pax7* mutant muscle, it is clear that SP cells and satellite cells represent distinct cell populations. Furthermore, the absence of muscle differentiation and the ~10-fold increased hematopoietic potential of *Pax7*$^{-/-}$ muscle-derived stem cells suggest the hypothesis that the progenitors for satellite cells are present within the muscle SP fraction. According to this model, upregulation of *Pax7* in muscle-derived pluripotent stem cells induces satellite cell specification by restricting alternate developmental programs (Figure 7). Alternately, muscle SP cells and satellite cells may represent independent cell populations. Inherent differences in the compositions of the SP fractions of wild-type and *Pax7*$^{-/-}$ muscle could explain the increased hematopoietic differentiation in mutant cultures.

Pax7 belongs to a family of genes that encode paired-box-containing transcription factors involved in the control of developmental processes (Jostes et al., 1990; Schafer et al., 1994). Different members of the Pax-family of transcription factors appear to regulate the development and differentiation of diverse cell lineages during embryogenesis (Noll, 1993; Strachan and Read, 1994; Mansouri et al., 1996a, 1996b, 1999). *Pax7* and the closely related *Pax3* gene belong to a paralogous subgroup of Pax genes based on similar protein structures and partially overlapping expression patterns during mouse embryogenesis (Jostes et al., 1990; Goulding et al., 1991). Interestingly, the *Pax3* gene plays an essential role in regulating the developmental program of embryonic myoblasts (Maroto et al., 1997; Tajbakhsh et al., 1997).

Pax7 and Pax3 proteins both bind identical sequence-specific DNA elements, which suggests that they regulate similar sets of target genes (Schafer et al., 1994). Furthermore, increased expression and gain-of-function mutations in both Pax3 and Pax7 are associated with the development of alveolar rhabdomyosarcomas, indicating that both molecules regulate similar activities in myogenic cells (Bennicelli et al., 1999). However, *Pax7* but not *Pax3* is expressed in adult human primary myoblasts (Schafer et al., 1994). Interestingly, differential expression of alternately spliced Pax7 transcripts correlates with muscle regenerative efficiency in different strains of mice (Kay et al., 1995, 1998). Although the Pax3 and Pax7 proteins are structurally similar, their different spatial-temporal patterns of expression suggest that they regulate myogenesis in distinct cell types during development.

Splotch (*Sp*) mice, lacking a functional *Pax3* gene, do not survive to term and fail to form limb muscles as the result of impaired migration of *Pax3*-expressing cells originating from the somite (Daston et al., 1996; Tremblay et al., 1998; reviewed in Borycki and Emerson, 1997). Compound mutant *Sp/Myf5*$^{-/-}$ mice do not express *MyoD* in their somites, suggesting that Myf5 and Pax3 function upstream of MyoD in myogenic determination (Tajbakhsh et al., 1997). Moreover, forced expression of *Pax3* induces *MyoD* expression and subsequent myogenesis in nonmuscle tissues from avian embryos (Maroto et al., 1997). However, ectopic expression of *Pax3* in C2C12 myoblasts efficiently inhibits myogenic differentiation (Epstein et al., 1995). In addition, co-expression of *MyoD* and *Pax3* is not observed in the mouse myotome (Williams and Ordahl, 1994). Therefore, Pax3 was suggested to function as an indirect upstream factor, which induced migration or other cellular changes to facilitate subsequent induction of *MyoD* transcription (reviewed in Borycki and Emerson, 1997).

Recent work suggests that Pax3 functions together with the Six1 transcription factor and the transcriptional co-regulators Eya2 and Dach2 to regulate the proliferation of pre-muscle masses in the somite (Heanue et al., 1999). *Pax3* and *Dach2* are co-expressed in the somite and appear to participate in a positive regulatory feedback loop. Ectopic expression experiments reveal that combinations of either Dach2 and Pax3 or Eya2 and Six1 synergize to induce myogenesis (reviewed in Relaix and Buckingham, 1999). It is interesting to speculate that an analogous regulatory network functions together with Pax7 in the specification of adult satellite cells.

Several genes have been suggested to represent direct targets for transcriptional regulation by Pax family members. Pax3 is believed to regulate *c-Met* transcription required to mediate the migration of somitic limb muscle precursors (Epstein et al., 1996). The c-Met receptor is expressed in quiescent satellite cells and is thought to activate satellite cells in response to hepatocyte growth factor (Allen et al., 1995; Cornelison and Wold, 1997). Thus, Pax7 may control the activation and migration of satellite cell precursors as a function of c-Met activity.

Regulatory elements in the neural cell adhesion molecule (NCAM) promoter are responsive to four Pax proteins, including Pax3 and Pax7 (Holst et al., 1997). NCAM is a member of the immunoglobulin superfamily of transmembrane proteins and has been implicated in the migration and differentiation of neural crest cells (Cunningham et al., 1987). Downregulation of NCAM in the developing neural crest of Pax7 mutant animals may be implicated in the dysgenesis of neural crest derivatives reported previously (Mansouri et al., 1996b). NCAM is

also expressed in activated satellite cells and myogenic precursor cells during muscle regeneration (Hurko and Walsh, 1983; Bischoff, 1994). NCAM may thus represent an important Pax7 target gene in satellite cells. Identification of transcriptional targets for Pax7 in the satellite cell lineage will be important for elucidating the mechanisms responsible for satellite cell specification, self-renewal, and activation.

Our experiments demonstrate that *Pax7*$^{-/-}$ muscle-derived pluripotent stem cells display almost an order of magnitude increase in hematopoietic potential (Figure 6I). Therefore, induction of *Pax7* in muscle-derived pluripotent stem cells appears to induce myogenic specification by restricting alternate developmental programs (Figure 7). This lineage-restricting function of Pax7 appears analogous to the role of Pax5 in regulating B cell development. Pro-B cells lacking Pax5 abnormally give rise to mature T cells expressing α/β-T cell receptors. Pax5 thus suppresses alternate lineage choices of B cell progenitors in a cell-autonomous manner (Nutt et al., 1999; Rolink et al., 1999).

Muscle satellite cells and embryonic muscle are believed to be derived from distinct progenitors during development (reviewed in Seale and Rudnicki, 2000). Indeed, the presence of grossly normal skeletal muscle in *Pax7*$^{-/-}$ mice, which completely lack satellite cells, underscores the assertion that embryonic myoblasts and myogenic satellite cells develop independently. The continued presence of muscle SP cells in *Pax7*$^{-/-}$ mice has important implications for the origin of satellite cells and the mechanism responsible for their self-renewal. SP cells may form a reservoir of satellite cell progenitors, which differentiate into myogenic satellite cells during the latter stages of embryonic muscle development and persist in adult skeletal muscle to maintain steady-state numbers of satellite cells. In addition, it remains possible that satellite cells themselves undergo self-renewal or that myogenic precursor cells de-differentiate to contribute to the satellite cell population.

Recent work suggests that satellite cells are derived from endothelial precursors associated with the embryonic vasculature (De Angelis et al., 1999). Therefore, an interesting possibility is that progenitors associated with the embryonic vasculature either directly or indirectly give rise to satellite cells at embryonic times, which reflects the vascularization of the tissue. Moreover, putative vasculature-associated precursors may continue to give rise to pluripotent stem cells in adult muscle.

The pluripotent nature of adult stem cells isolated from diverse tissues raises the possibility of combined gene and stem-cell therapy for a variety of degenerative diseases, including muscular dystrophy. For example, ectopic expression of *Pax7* and *dystrophin* in pluripotential stem cells may result in the generation of high numbers of pre-satellite cells that would efficiently contribute to the damaged muscle of Duchenne patients. Indeed, ectopic expression of different members of the Pax gene family may direct the development of pluripotential stem cells into a range of discrete cell lineages. Detailed analysis of the potential of muscle stem cells ectopically expressing developmental control genes will elucidate the utility of such an approach.

Experimental Procedures

Molecular Cloning of Pax7 and Expression Analysis

RDA was performed as described by Hubank and Schatz (1994). Satellite cell-derived myoblast cDNA was subtracted twice against mouse embryonic fibroblast (MEF) cDNA (1:100; 1:400) and once against skeletal muscle cDNA (1:400) to generate the final difference products. The full-length mouse cDNA for *Pax7* was isolated by screening an adult mouse skeletal muscle library (Clontech) using the RDA clone as a probe (Maniatis et al., 1982).

Total RNA was extracted as previously described (Chomczynski and Sacchi, 1987). Northern blot analysis of 20 μg of total RNA from tissue or cell cultures was performed according to Maniatis et al. (1982). In situ hybridization for *Pax7* mRNA was performed as described by Braissant and Wahli (1998). Sections were counterstained with 100 μg/mL PI (Sigma) in PBS for 10 min at room temperature. Three different Pax7 sequences from the full-length cDNA were used as cRNA probes: Pax7-Sal1, nts 150–1600; dp3–7, nts 4200–4700; and Pax7-Cla1, nts 515–1500.

Myoblast and Stem-Cell Culture

Primary muscle cultures were isolated as in Sabourin et al. (1999). Primary MEFs were isolated from 13.5-day-old Balb/c mouse embryos (Robertson, 1987). Single muscle fibers were isolated from hind limb skeletal muscles, as described by Cornelison and Wold (1997). Individual fibers were cultured in methocult GF M3434 containing 15% FBS, 1% BSA, 10^{-4} M 2-mercaptoethanol, 10 μg/mL pancreatic insulin, 200 μg/mL transferrin, 50 ng/mL SCF, 10 ng/mL IL-3, 10 ng/mL IL-6, and 3 units/mL EPO (Stem Cell Technologies) from 48 hr to 10 days.

For hematopoietic colony-forming assays, cell suspensions were derived from skeletal muscle by digestion in 0.4% collagenase Type A (Roche)/DMEM for 1.5 hr at 37°C, filtered (74 μm; Costar Netwell), and resuspended at 100 cells/μl in 10% horse serum/DMEM. Approximately 10,000 cells were cultured in 3 ml of methocult (Stem Cell Technologies) for 14 days.

FACS

Hoechst staining and FACS analysis were performed essentially as described previously (Goodell et al., 1996). FACS was performed on a Becton-Dickinson FacStar flow cytometer equipped with dual lasers. Hoechst dye was excited at 350 nm, and its fluorescence was measured at two wavelengths using a 424BP44 filter (blue emission) and a 650LP filter (red emission). A 640 DMSP mirror was used to separate wavelengths.

Immunocytochemistry and Electron Microscopy

Primary cell cultures or colonies picked from methocult medium were fixed and stained as described elsewhere (Sabourin et al., 1999) using anti-c-Met SP260 (Santa Cruz), anti-desmin DE-U-10 (DAKO), anti-mouse Ly-6G (clone RB6-8C5) (Pharmingen), anti-mouse integrin α_M (M1/70) (Pharmingen), and MF20 mAb (anti-MHC).

Gastrocnemius muscle was prepared for TEM by overnight fixation at 4°C in 2% gluteraldehyde/0.1 M cacodylate (pH 7.4) and processed using standard procedures as described by Kablar (1995). Randomly chosen fields were viewed with a Jeol 1200EX Biosystem TEM. Diaphragm and tibialis anterior muscles were prepared for HE staining as described by Bancroft and Stevens (1990).

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References

Allen, R.E., Sheehan, S.M., Taylor, R.G., Kendall, T.L., and Rice, G.M. (1995). Hepatocyte growth factor activates quiescent skeletal muscle satellite cells in vitro. J. Cell. Physiol. *165*, 307–312.

Appell, H.J., Forsberg, S., and Hollmann, W. (1988). Satellite cell activation in human skeletal muscle after training: evidence for muscle fiber neoformation. Int. J. Sports Med. *9*, 297–299.

Bancroft, J.D., and Stevens, A. (1990). Theory and Practice of Histological Techniques, Third Edition (Edinburgh and New York: Churchill Livingstone).

Bennicelli, J.L., Advani, S., Schafer, B.W., and Barr, F.G. (1999). PAX3 and PAX7 exhibit conserved *cis*-acting transcription repression domains and utilize a common gain of function mechanism in alveolar rhabdomyosarcoma. Oncogene *18*, 4348–4356.

Bischoff, R. (1994). The satellite cell and muscle regeneration. In Myogenesis, A.G. Engel and C. Franszini-Armstrong, eds. (New York: McGraw-Hill), pp. 97–118.

Borycki, A.G., and Emerson, C.P. (1997). Muscle determination: another key player in myogenesis? Curr. Biol. *7*, R620–R623.

Braissant, O., and Wahli, W. (1998). Differential expression of peroxisome proliferator-activated receptor-alpha, -beta, and -gamma during rat embryonic development. Endocrinology *139*, 2748–2754.

Chomczynski, P., and Sacchi, N. (1987). Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. Anal. Biochem. *162*, 156–159.

Cornelison, D.D., and Wold, B.J. (1997). Single-cell analysis of regulatory gene expression in quiescent and activated mouse skeletal muscle satellite cells. Dev. Biol. *191*, 270–283.

Cunningham, B.A., Hemperly, J.J., Murray, B.A., Prediger, E.A., Brackenbury, R., and Edelman, G.M. (1987). Neural cell adhesion molecule: structure, immunoglobulin-like domains, cell surface modulation, and alternative RNA splicing. Science *236*, 799–806.

Daston, G., Lamar, E., Olivier, M., and Goulding, M. (1996). Pax-3 is necessary for migration but not differentiation of limb muscle precursors in the mouse. Development *122*, 1017–1027.

De Angelis, L., Berghella, L., Coletta, M., Lattanzi, L., and Zanchi, M., Cusella-De Angelis, M.G., Ponzetto, C., and Cossu, G. (1999). Skeletal myogenic progenitors originating from embryonic dorsal aorta coexpress endothelial and myogenic markers and contribute to postnatal muscle growth and regeneration. J. Cell Biol. *147*, 869–878.

Epstein, J.A., Lam, P., Jepeal, L., Maas, R.L., and Shapiro, D.N. (1995). Pax3 inhibits myogenic differentiation of cultured myoblast cells. J. Biol. Chem. *270*, 11719–11722.

Epstein, J.A., Shapiro, D.N., Cheng, J., Lam, P.Y., and Maas, R.L. (1996). Pax3 modulates expression of the c-Met receptor during limb muscle development. Proc. Natl. Acad. Sci. USA *93*, 4213–4218.

Fleming, T.J., Fleming, M.L., and Malek, T.R. (1993). Selective expression of Ly-6G on myeloid lineage cells in mouse bone marrow. RB6–8C5 mAb to granulocyte-differentiation antigen (Gr-1) detects members of the Ly-6 family. J. Immunol. *151*, 2399–2408.

George-Weinstein, M., Foster, R.F., Gerhart, J.V., and Kaufman, S.J. (1993). In vitro and in vivo expression of alpha 7 integrin and desmin define the primary and secondary myogenic lineages. Dev. Biol. *156*, 209–229.

Gibson, M.C., and Schultz, E. (1983). Age-related differences in absolute numbers of skeletal muscle satellite cells. Muscle Nerve *6*, 574–580.

Goodell, M.A., Brose, K., Paradis, G., Conner, A.S., and Mulligan, R.C. (1996). Isolation and functional properties of murine hematopoietic stem cells that are replicating in vivo. J. Exp. Med. *183*, 1797–1806.

Goodell, M.A., Rosenzweig, M., Kim, H., Marks, D.F., DeMaria, M., Paradis, G., Grupp, S.A., Sieff, C.A., Mulligan, R.C., and Johnson, R.P. (1997). Dye efflux studies suggest that hematopoietic stem cells expressing low or undetectable levels of CD34 antigen exist in multiple species. Nat. Med. *3*, 1337–1345.

Goulding, M.D., Chalepakis, G., Deutsch, U., Erselius, J.R., and Gruss, P. (1991). Pax-3, a novel murine DNA binding protein expressed during early neurogenesis. EMBO J. *10*, 1135–1147.

Grounds, M.D., and Yablonka-Reuveni, Z. (1993). Molecular and cell biology of skeletal muscle regeneration. Mol. Cell. Biol. Hum. Dis. *3*, 210–256.

Gussoni, E., Soneoka, Y., Strickland, C.D., Buzney, E.A., Khan, M.K., Flint, A.F., Kunkel, L.M., and Mulligan, R.C. (1999). Dystrophin expression in the mdx mouse restored by stem cell transplantation. Nature *401*, 390–394.

Heanue, T.A., Reshef, R., Davis, R.J., Mardon, G., Oliver, G., Tomarev, S., Lassar, A.B., and Tabin, C.J. (1999). Synergistic regulation of vertebrate muscle development by Dach2, Eya2, and Six1, homologs of genes required for Drosophila eye formation. Genes Dev. *13*, 3231–3243.

Holst, B.D., Wang, Y., Jones, F.S., and Edelman, G.M. (1997). A binding site for Pax proteins regulates expression of the gene for the neural cell adhesion molecule in the embryonic spinal cord. Proc. Natl. Acad. Sci. USA *94*, 1465–1470.

Hubank, M., and Schatz, D.G. (1994). Identifying differences in mRNA expression by representational difference analysis of cDNA. Nucleic Acids Res. *22*, 5640–5648.

Hurko, O., and Walsh, F.S. (1983). Human fetal muscle-specific antigen is restricted to regenerating myofibers in diseased adult muscle. Neurology *33*, 737–743.

Irintchev, A., Zeschnigk, M., Starzinski-Powitz, A., and Wernig, A. (1994). Expression pattern of M-cadherin in normal, denervated, and regenerating mouse muscles. Dev. Dyn. *199*, 326–337.

Jackson, K.A., Mi, T., and Goodell, M.A. (1999). Hematopoietic potential of stem cells isolated from murine skeletal muscle. Proc. Natl. Acad. Sci. USA *96*, 14482–14486.

Jostes, B., Walther, C., and Gruss, P. (1990). The murine paired box gene, *Pax7*, is expressed specifically during the development of the nervous and muscular system. Mech. Dev. *33*, 27–37.

Kablar, B. (1995). Structural study on the appearance of innervation in the stomach of mouse and rat embryos. Tissue Cell *27*, 309–315.

Kay, P.H., Mitchell, C.A., Akkari, A., and Papadimitriou, J.M. (1995). Association of an unusual form of a *Pax7*-like gene with increased efficiency of skeletal muscle regeneration. Gene *163*, 171–177.

Kay, P.H., Harmon, D., Fletcher, S., Robertson, T., Ziman, M., and Papadimitriou, J.M. (1998). Pax7 includes two polymorphic homeoboxes which contain rearrangements associated with differences in the ability to regenerate damaged skeletal muscle in adult mice. Int J. Biochem. Cell Biol. *30*, 261–269.

Leenen, P.J., de Bruijn, M.F., Voerman, J.S., Campbell, P.A., and van Ewijk, W. (1994). Markers of mouse macrophage development detected by monoclonal antibodies. J. Immunol. Methods *174*, 5–19.

Maniatis, T., Fritsch, E.F., and Sambrook, J. (1982). Molecular Cloning: A Laboratory Manual (Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press).

Mansouri, A., Hallonet, M., and Gruss, P. (1996a). Pax genes and their roles in cell differentiation and development. Curr. Opin. Cell Biol. *8*, 851–857.

Mansouri, A., Stoykova, A., Torres, M., and Gruss, P. (1996b). Dysgenesis of cephalic neural crest derivatives in Pax7–/– mutant mice. Development *122*, 831–838.

Mansouri, A., Goudreau, G., and Gruss, P. (1999). Pax genes and their role in organogenesis. Cancer Res. *59*, 1707s–1710s.

Maroto, M., Reshef, R., Munsterberg, A.E., Koester, S., Goulding, M., and Lassar, A.B. (1997). Ectopic Pax-3 activates MyoD and Myf-5 expression in embryonic mesoderm and neural tissue. Cell *89*, 139–148.

Megeney, L.A., Kablar, B., Garrett, K., Anderson, J.E., and Rudnicki, M.A. (1996). MyoD is required for myogenic stem cell function in adult skeletal muscle. Genes Dev. *10*, 1173–1183.

Morlet, K., Grounds, M.D., and McGeachie, J.K. (1989). Muscle precursor replication after repeated regeneration of skeletal muscle in mice. Anat. Embryol. *180*, 471–478.

Noll, M. (1993). Evolution and role of Pax genes. Curr. Opin. Genet. Dev. *3*, 595–605.

Nutt, S.L., Heavey, B., Rolink, A.G., and Busslinger, M. (1999). Commitment to the B-lymphoid lineage depends on the transcription factor Pax5. Nature *401*, 556–562.

Relaix, F., and Buckingham, M. (1999). From insect eye to vertebrate muscle: redeployment of a regulatory network. Genes Dev. *13*, 3171–3178.

Robertson, E.J. (1987). Embryo-derived stem cell lines. In Teratocarcinomas and Embryonic Stem Cells: A Practical Approach, E.J. Robertson, ed. (Oxford: IRL Press Ltd.), pp. 71–112.

Rolink, A.G., Nutt, S.L., Melchers, F., and Busslinger, M. (1999). Long-term in vivo reconstitution of T-cell development by Pax5-deficient B-cell progenitors. Nature *401*, 603–606.

Rosenblatt, J.D., Yong, D., and Parry, D.J. (1994). Satellite cell activity is required for hypertrophy of overloaded adult rat muscle. Muscle Nerve *17*, 608–613.

Sabourin, L.A., Girgis-Gabardo, A., Seale, P., Asakura, A., and Rudnicki, M.A. (1999). Reduced differentiation potential of primary MyoD–/– myogenic cells derived from adult skeletal muscle. J. Cell Biol. *144*, 631–643.

Schafer, B.W., Czerny, T., Bernasconi, M., Genini, M., and Busslinger, M. (1994). Molecular cloning and characterization of a human PAX-7 cDNA expressed in normal and neoplastic myocytes. Nucleic Acids Res. *22*, 4574–4582.

Schultz, E., and Jaryszak, D.L. (1985). Effects of skeletal muscle regeneration on the proliferation potential of satellite cells. Mech. Ageing Dev. *30*, 63–72.

Schultz, E., Jaryszak, D.L., and Valliere, C.R. (1985). Response of satellite cells to focal skeletal muscle injury. Muscle Nerve *8*, 217–222.

Seale, P., and Rudnicki, M.A. (2000). A new look at the origin, function, and "stem-cell" status of muscle satellite cells. Dev. Biol. *218*, 115–124.

Sicinski, P., Geng, Y., Ryder-Cook, A.S., Barnard, E.A., Darlison, M.G., and Barnard, P.J. (1989). The molecular basis of muscular dystrophy in the mdx mouse: a point mutation. Science *244*, 1578–1580.

Strachan, T., and Read, A.P. (1994). PAX genes. Curr. Opin. Genet. Dev. *4*, 427–438.

Tajbakhsh, S., Rocancourt, D., Cossu, G., and Buckingham, M. (1997). Redefining the genetic hierarchies controlling skeletal myogenesis: Pax-3 and Myf-5 act upstream of MyoD. Cell *89*, 127–138.

Tremblay, P., Dietrich, S., Mericskay, M., Schubert, F.R., Li, Z., and Paulin, D. (1998). A crucial role for Pax3 in the development of the hypaxial musculature and the long-range migration of muscle precursors. Dev. Biol. *203*, 49–61.

Williams, B.A., and Ordahl, C.P. (1994). Pax-3 expression in segmental mesoderm marks early stages in myogenic cell specification. Development *120*, 785–796.